**Issue of inventiveness**

CLAIM 1: OBTAINING THE HUMAN SMN RT-PCR PRODUCT

Concerning claim 1, Office Action (p. 9) states that:

> "Routine optimization is not considered inventive and no evidence has been presented that the selection of the claimed RT and PCR reaction conditions was other than routine or that the results should be considered unexpected in any ways as compared to the closest prior art.
>
> Therefore, the person of ordinary skill in the art, interested in obtaining a human SMN RT-PCR product, would have been motivated to perform the RT-PCR method taught by Wirth using any primer set derived from the known human SMN gene sequence, as suggested by Powell, Lefebvre, and Buck, under optimized reaction conditions, as suggested by Innis, thus resulting in the instantly claimed method."

Precedent

Examples of precedent cases related to this applicant's appropriate combining of prior known means, well known compounds or devices for which U.S. patents were granted.

- Nguyen, U.S Patent No. 5,624,831 for a method allowing to stabilize the enzymatic activity of immobilized acetylcholinesterase by combining well known compounds of gelatin or albumin and trehalose to stabilize the enzymatic activity of immobilized acetylcholinesterase (well known enzyme). The same compounds were used in a different U.S. Patent No. 4,324,858, and in the publication on "Extraordinary stability of enzymes dried in trehalose" (C. Colaco, et al., Biotechnology, 1992, 9, 1007-1013).

- Nguyen, U.S. Patent No. 6,639,058 for a method of dissolving preformed beta-amyloid peptide fibrils in-vitro by poly-L-lysine. Poly-L-lysine, known compound used for a variety of other purposes, was used in this invention to dissolve preformed beta-amyloid peptide fibrils found in the brains of people with Alzheimer's disease; previously used to bind with protein (histone) and DNA in order to analyze the complexation between DNA and histone in chromatin (Hsueh Jei Li et al., Biochemistry, 1973, 12, 9). Known device used: Electron microscopy.

- I am the same inventor in Nguyen et al., U.S. Patent No. 6,924,102 (U.S. Publication No. 20030049627) of a procedure that allows the measurement of specific mRNA for the molecular diagnosis of autosomal recessive spinal muscular atrophy (SMA) – mentioned in Office Action -- combining of the well known means and techniques of the field of molecular biology as described by Powell, Gruber, Feuerstein, Bruce, and Lefebvre, under optimized reaction conditions, resulted in the human SMN RT-PCR product.

Explanations regarding U.S. Patent No. 6,924,102 that reflects the related issue of inventiveness.

*Background of SMA disease and the development of a quantitative molecular diagnostic for SMA*

Spinal muscular atrophy (SMA) is a neuromuscular disorder characterized by a degeneration of motor neurons from the ventral horn of the spinal cord, leading to symmetrical paralysis of voluntary muscles with muscular atrophy. To date, definitive treatment information about SMA has not been sought consistently. The survival motor neuron (SMN) gene is found to be partially deleted or mutated in over 98% of SMA patients and therefore considered the SMA determining gene.

*Duplication of SMN gene and the need for a quantitative diagnostic method*

The SMN gene duplicates into highly homologous copies – SMNT (telemoric) and SMNC (centromeric). Only homozygous absence of SMNT exons 7 and 8 is responsible for SMA disease. For the diagnosis of SMA, Lefebvre et al. uses the SSCP technique to analyze SMN gene which means working at the DNA level to detect the presence or absence of homozygous deletions of SMNT exons 7 and 8 or only exon 7 (Lefebvre et al., Cell, 1995, **80**, 155-165). However, this qualitative diagnostic method does not allow the identification of

heterozygous deletions of SMNT exons 7 and 8 (SMA carriers). This is due to the highly complex problem caused by the duplication of SMN gene. Only a quantitative diagnostic method that permits the precise counting of the number of exons 7 and 8 in SMNT and SMNC would allow the detection of SMA carriers. Thus, to identify SMA carriers, a few researchers (McAndrew et al., American Journal of Human Genetics, 1997, **60**, 1411-1422; Chen et al., American Journal of Medical Genetics, 1999, **85**, 463-469; Ogino et al., Journal of Molecular Diagnostics, 2001, **3**, 150-157) have tried to use the quantitative PCR technique from the SMN gene, which means working at the DNA level to try to count the number of SMNT and SMNC. However, in order to avoid inaccuracies in the results, this quantitative PCR technique at the DNA level needs a great deal of techniques precautions such as the controls prepared by the same extraction method, the preflashing of the film and monitoring exposure times to ensure the linearity of film response for autoradiography. Furthermore, this method is not suited for diagnostic for purpose to be used in clinical laboratories.

*Non-obviousness*

As seen above, researchers have continued to focus their study at the DNA level. The problem to detect SMA carriers thus remains unresolved.

-What is not obvious is to conceptualize a different approach to develop a quantitative diagnostic method. This is not a matter of using known techniques. Previously, researchers have focused their work at the DNA level, using well known techniques of the field of molecular biology as described by Powell, Gruber, Bruce, Lefebvre and yet they have been unable to adequately resolve the problem of detecting SMA carriers.

-What is not obvious is to figure out the right place to focus on, in order to develop an accurate quantitative diagnostic method. The difficulty lies in the highly complex problem of

gene duplication inherent in SMA disease. Thus, what is not obvious is Nguyen's conceptualization of where to focus on (U.S. Patent No. 6,924,102; U.S. Publication No. 20030049627). Nguyen's choice is based on the concept originating from the relationship between the DNA and protein via the intermediary phase of mRNA.

DNA gives mRNA by transcription process, and in turn, mRNA gives protein by translation process:

$$\text{DNA} \xrightarrow[\text{Transcription}]{} \text{mRNA} \xrightarrow[\text{Translation}]{} \text{protein}$$

Therefore, normally, anything that happens at the DNA level will be reflected at the mRNA level and at the protein level. If there is a problem such as mutation or deletion at the DNA level, this problem will also be found at the mRNA level, and the protein will be non-functional thus leading to disease. However, in certain cases what happens at the DNA level cannot be found at the mRNA level due to mRNA editing (S. Maas, A. Rich, Bioessays, 2000, **22**, 790-802) or due to stop codon suppression (D.N. Robinson, L. Cooley, Development, 1997, **124**, 1405-1417; S.L. Alam, N.M. Wills, J.A. Ingram, et al. J. Mol. Biol., 1999, **288**, 837-852); either case leads to a functional protein. This is the reason why it is important to consider working at the mRNA level.

Regarding SMA disease, 98% of SMA patients carry homozygous deletions of exons 7 and 8 or only exon 7 of SMNT, either because of conversion of sequences in SMNT to those in SMNC, or as a result of SMNT deletions (Lefebvre et al., Cell, 1995, **80**, 155-165).

Nguyen (Patent No. 6,924,102; Publication No. 20030049627) thus decided to pursue to place the work focus at the mRNA level based on the observation that the SMNT transcripts (SMNT-mRNA) were absent and that the SMNC transcripts (SMNC-mRNA) were solely present in SMA patients lacking the SMN gene on both mutant chromosomes, while control

individuals expressed both RNA transcripts, SMNT-mRNA and SMNC-mRNA (Lefebvre et al., Cell, 1995, **80**, 155-165). Furthermore, the SMNC transcripts, not the SMNT transcripts, may undergo alternative splicing of exon 7 to produce transcripts lacking this exon.

*Non-obvious next steps*

Once Nguyen decided that the work should be done at the mRNA level, what is not obvious is to identify the approach to develop a quantitative diagnostic method (Patent No. 6,924,102; Publication No. 20030049627).

Jong et al. (Journal of Neurological Sciences, 2000, 173, 147-153), in a semi-quantitative research method using Image Analysis System, analyzed the mRNA transcripts of the SMN gene in the peripheral blood mononuclear cells of normals, carriers and SMA patients:

Total RNA
---
↓
RT process using **RANDOM** primer PolydT which results in cDNA
**NON-SPECIFIC**
---
↓
PCR process using different specific primers and the biotin-labeled probes to locate the SMN fragments
----- -- Exon 7-- -------- ----------
↓

After locating which ones are SMN fragments (by Southern blot analysis and by using antibodies anti-biotin), performing the sequencing to detect the presence of exon 7 in the above mentioned different PCR fragments.

Then, they examined lighter or darker shades of bands 355 bp (presence of exon 7) and 301 bp (absence of exon 7), and 419 bp (presence of exon 7) and 365 bp (absence of exon 7) of PCR fragments. Such examination of the lighter or darker shades of the PCR fragments in 3% agarose gels does not give accurate results; examination of lighter or darker shades of the bands that are so close to each other (355 bp and 301 bp; 419 bp and 365 bp) does not give precise

results (see figures 2.B and 2.C page 150, Jong et al., Journal of Neurological Sciences, 2000, **173**, 147-153). Jong's work is an analysis in basic research using a semi-quantitative research method; it is not for diagnostic purpose.

The above-mentioned example shows that it is not obvious to identify an appropriate approach to develop a quantitative diagnostic method for SMA disease.

Nguyen adopted a totally different approach (U.S. Patent No. 6,924,102; U.S. Publication No. 20030049627) summarized as follow:

Total RNA
---
↓
RT process using **SPECIFIC** primer (SEQ ID NO: 1) which recognizes the **SPECIFIC** SMN-mRNA; this results in **SMN-cDNA**

---------- SMN-cDNA
↓
PCR process using the **SPECIFIC** primers (SEQ ID NO: 3) and (SEQ ID NO: 4) to amplify the sequence fragments between exons 5 to 8

Exon 5 ---------- Exon 8
↓

Construction of the probes:

From the obtained fragments 5 to 8 by PCR:

- using the primers (SEQ ID NO: 7) and (SEQ ID NO: 8) to amplify a portion of exon 7
- using the primers (SEQ ID NO: 9) and (SEQ ID NO: 4) to amplify a portion of exon 8

The portion of exon 7 is labeled in two ways with radioactive label and with biotin label (non radioactive) to get probe 1

The portion of exon 8 is labeled in two ways with radioactive label and with biotin label (non radioactive) to get probe 2

The probes 1 and 2 are used to identify the presence or absence of exons 7 and 8 in normal subjects and SMA patients.

Nguyen's quantitative diagnostic method is based on the use of SPECIFIC probes to DIRECTLY identify the presence or absence of exons 7 and 8 in different types of subjects (Patent No. 6,924,102; Publication No. 20030049627). As of the issue date of the patent, Nguyen's quantitative SMA diagnostic method at the **mRNA level** is the **only** diagnostic method that permits such precise counting of the number of exons 7 and 8 by measuring the **quantity** of radioactive rays emitted from the radioactive labeled probes, and by measuring the **intensity** of the coloration of the solution by using the biotin labeled probes in ELISA method (Patent No. 6,924,102; Publication No. 20030049627). No one before has developed the method of synthesizing the probes specifically for SMA diagnostic purpose – probes directed to the deletion regions for the detection of the presence or absence of the nucleic acids (examiner acknowledges that Jong et al. do not teach this, p.11 Office Action).

*How to quantify: Two ways of measuring*

1/ Use of radioactive labeled probes ($^{32}$p dCTP labeled nucleotide probes)

After immobilizing the PCR products on the nylon membrane, we perform the hybridization of the immobilized PCR products with the radioactive labeled probe 1 and probe 2. During this step, if the immobilized PCR products contain exon 7 and/or exon 8, the radioactive labeled probes 1 and/or 2 will bind to the immobilized PCR products at the position of exon 7 and/or exon 8. We thus detect the presence of the hybridized probes, which means the binding of the probes to the immobilized PCR products by autoradiography; we then quantify the amount of hybridized probes 1 and 2 by means of BioImager device by measuring the quantity of radioactivity rays emitted from the radioactive labeled probes 1 and 2. The quantity of radioactive rays emitted is proportional to the amount of radioactive labeled probes 1 and 2, and

consequently proportional to the number of exon 7 and/or exon 8 present in the mRNA; this way allows us to count the number of exon 7 and/or 8 present in the mRNA.

2/ Use of biotin labeled probes in ELISA procedure for measurement of mRNA

During the hybridization of the PCR products (containing the digoxigenin-dUTP) with the biotin labeled nucleotide probe 1 and probe 2, if the PCR products contain exon 7 and exon 8, the biotin labeled probes 1 and 2 will bind to the PCR products at the position of exon 7 and/or exon 8. After this step, the following was performed:

- Immobilization of the hybridized PCR products on streptavidin coated microtitration plates. During this step, the streptavidin will bind to the biotin molecules of the biotin labeled probes 1 and 2.
- Adding the peroxidase-conjugated anti-digoxigenin antibodies. This compound will bind to the digoxigenin-dUTP present in the hybridized PCR products.
- Adding the peroxidase substrates which are $H_2O_2$ and chromogene (tetramethyl benzidine).
- Quantification of the amount of biotin-labeled probes 1 and 2 by means of a microplate reader device by measuring the transmitted light emitted from the chromogene (tetramethyl benzidine) which is one of the substrates of peroxidase enzyme. The quantity of transmitted light emitted from the chromogene is proportional to the amount of biotin labeled probes 1 and 2, and consequently proportional to the number of exon 7 and/or exon 8 present in the mRNA; this way thus allows the counting of the number of exon 7 and/or exon 8 present in the mRNA.

As shown in the above description, it is not obvious to identify appropriate steps applicable for the selected approach in order to develop a quantitative diagnostic method for SMA disease. Jong's work arrived at a semi-quantitative method, not quantitative; and the purpose is not for diagnostic purpose.

The point is to determine what steps to take, then determine which techniques are needed to perform such tasks.

*Contribution over the art*

The state of the art is the qualitative diagnostic method of SMA at the DNA level. Nguyen's contribution over the art is the quantitative diagnostic method at the mRNA level that permits the precise counting of the number of exons 7 and 8 (U.S. Patent No. 6,924,102; U.S. Publication No. 20030049627). The innovation is the use of specific probes to directly identify the presence or absence of exons 7 and 8: No one before has developed the method of synthesizing the probes specifically for SMA diagnostic purpose.

The point is not just to develop a quantitative molecular diagnostic method of SMA. The contribution over the art is the selection of the appropriate approach and combination of appropriate techniques that results in the development of this quantitative molecular diagnostic method of SMA which is safe, easy to handle and automated, easy to interpret, not costly and can be used widely in clinical laboratories. In addition, the contribution over the art is that the application offers two ways of measuring – by means of BioImager device using the radioactive labeled probes and by means of a microplate reader device in ELISA procedure using the biotin labeled probes; selecting which way depends on the type of equipment available at the laboratory.

Relating to the present application

The above mentioned explanations reflect a precedent for the process of the construction of the expression plasmids in order to produce full-length SMN recombinant protein in the present application.

- Present application: Result of continuing research work done beyond his invention in U.S. Patent No. 6,924,102 (Nguyen et al.) in order to contribute over the art.

*Background of SMA disease and the construction of the expression plasmids for SMN recombinant protein*

The SMN gene encodes a 38-kDa protein ubiquitously expressed, the SMN protein, which reveals no significant sequence homology with other proteins. Its exact function is actually unknown. To date, there is no definitive treatment for SMA. SMN is an essential protein since a knockout mouse for the SMN gene is embryonically lethal, suggesting that SMN is the determining gene. Thus, the availability of full-length SMN protein is valuable for biochemical and biological analyses which may elucidate the molecular mechanism of SMA. This information will be useful for the development of a therapy for SMA.

*State of the art*

The state of the art is the method to obtain full-length SMN recombinant protein based on the isolation of SMN-cDNA clones from the cDNA library using the synthesized oligonucleotide probes which is very time-consuming and requires highly skilled personnel to perform.

**Contribution over the art**

The present application offers a new method to overcome the above mentioned technical problem. What is inventive and what a person of ordinary skill in the art would not do is to conceptualize a different approach in order to get full-length SMN recombinant protein most

easily and rapidly. This is not just a matter of using prior known means, well known compounds, well known devices, or well known techniques of the field of molecular biology as described by Wirth, Powell, Lefebvre, Buck, Innis, and Melki. As a matter of fact they have not indicated the way to resolve the technical problems to obtain full-length SMN recombinant protein in a fast and easy way.

Thus, what is inventive and what a person of ordinary skill in the art would not do is the present invention: The new procedure to **get the SMN constructs using the easiest and fastest method via the commercially available expression plasmid vectors in order to get full-length recombinant SMN protein in different expression systems.** This procedure consists of: 1) Performing the RT-PCR reactions from the total RNA in order to get the PCR products corresponding to the CDS (885 bp) of the SMN gene that encodes full-length SMN protein, SMN-cDNA (Claim 1); and 2) Ligating the SMN-cDNA PCR products to the selected commercially available expression plasmids vectors in order to get the SMN constructs capable of producing full-length recombinant SMN protein in different expression systems, such as baculovirus and bacterial expression systems (Claims 2 and 3).

What is inventive and what a person of ordinary skill in the art would not do is to figure out the specific, easiest and fastest procedure to focus on, in order to get the SMN constructs capable of producing rapid, high yield and stable full-length SMN recombinant protein.

Once this applicant decided that the work should be done via RT-PCR reactions from the total RNA, what is inventive and what a person of ordinary skill in the art would not do is to identify the RT-PCR conditions in order to get directly and specifically the PCR products corresponding to the CDS (885 bp) of the SMN gene that encodes full-length SMN protein: SMN-cDNA.

Once the SMN-cDNA is obtained, what is inventive and what a person of ordinary skill in the art would not do is to identify the approach in order to get the SMN constructs easily and rapidly. The point is to determine what steps to take, then determine which techniques are appropriate to perform the needed tasks.

*Not a task for a person of ordinary skill in the art to perform*

Reverse Transcription reaction in claim 1

In this applicant's work, the specific primer (SEQ ID NO: 1) was used to perform the reverse transcription reaction (RT) which recognizes the SPECIFIC SMN-mRNA resulting in SMN-cDNA – this is different from the use of oligo-dT primers taught by Wirth which results in cDNA NON-SPECIFIC. Powell's statement (quoted on page 6, Office Action) – "One of the advantages of using specific primers close to the site of modification is that cDNA synthesis and subsequent amplification may be performed on less than intact preparations of total RNA" – indicates the vague state of knowledge at the time. **Over Powell, this application indicates advancement toward a more precise piece of knowledge by demonstrating the main advantage of using specific primers for RT reaction (SEQ ID NO: 1)**: The main advantage is to get the SPECIFIC cDNA which results in obtaining the expected PCR products specifically and directly via PCR amplification. Wirth teaches RT reaction using oligo-dT primers at conditions of 37° C for 50 minutes (Office Action, p. 5), while this applicant uses specific primer (SEQ ID NO: 1) for RT reaction conducted at 42° C for 45 minutes according to the Tm of the specific primer (SEQ ID NO: 1) used.

*Not a task for a person of ordinary skill in the art to perform*

Polymerase Chain Reaction in claim 1

In response to the Office Action, clarifications regarding Polymerase Chain Reaction (PCR) amplification need to be first brought forth.

Concerning PCR amplification, the selection of the specific primers is based on the sequence of the target gene. Contrary to what is taught by Buck (Office Action p. 6, 7 and 11) – "...This clearly shows that every primer would have a reasonable expectation of success" (p. 7) – Buck's statement is inaccurate. The fact is that not all selected primers have the same probability of reasonable success. As a matter of fact, some of the selected primers have no success at all in PCR amplification! In general, for a given selected primer, besides the issue of selecting the specific primers, the success in getting the expected PCR product also depends on the PCR reaction conditions, such as time and/or temperature of the denaturation, and annealing and extension steps. **Contrary to Buck's teaching, this applicant selected the specific primers (SEQ ID NOS: 2 and 3), and optimized the conditions for PCR amplification by taking into account the specific properties (base composition, length and concentration) of the target gene and primer sequences.**

Concerning the number of cycles for PCR reaction, contrary to what is taught by Innis (Office Action p. 7 and 11-12) – "These conditions are: 25 - 35 cycles of 96°C for 15 seconds..." (Office Action p. 7), "The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimized" (Office Action p. 12) **Over Innis, this applicant teaches the number of cycles for PCR amplification which is determined by taking into account the objective of the research work.** If the objective is to get a high quantity of PCR products, the number of cycles of the PCR amplification would be 30

to 40 cycles. However, for research work using a quantitative method based on PCR amplification, the number of cycles of the PCR amplification would be 20 to 25 cycles in order to ensure quantitative measurements during the linear phase of the PCR amplification.

Regarding the Office Action' statement (p. 5, 6), for PCR amplification, Wirth et al. used primers different from the ones used in the present application (SEQ ID NOS: 2 and 3), it must be pointed out that the reason is because the primers selected by Wirth et al. were targeted to the exon 1 (between base pairs 19 – 36) and exon 8 (between base pairs 1177 – 1194) of the SMN gene for SMA diagnostic purpose. On the other hand, the objective of the present application is different because the research objective is to get the SMN protein (295 amino acids); therefore, the CDS (885 bp) of the SMN gene is needed. The primers for PCR amplification (SEQ ID NOS: 2 and 3) were therefore selected based on the sequence between base pairs 1 – 17 (SEQ ID NO: 2) and 864 - 885 (SEQ ID NO: 3) of the CDS of the SMN gene. Consequently, the PCR conditions used by Wirth et al. and this applicant have to be different; therefore, the difference between the number of cycles, 30 cycles by Wirth et al. and 35 cycles by this applicant, is minimal because the quantity of the PCR products is almost the same between 30 and 40 cycles, due to saturation of the PCR products between 30 and 40 cycles.

Clarifications regarding the primer sequences in U.S. Patent No. 6,924,102 and the present application

*Primer sequence selection in function of research objective*

One must be reminded that the selection of the primer sequences must be in function of the objective of the research work.

In regards to (c) (Office Action p. 10), for PCR amplification, SEQ ID NOS 3 and 4 used by Nguyen et al. are actually specified in U.S. Patent No. 6,924,102 – this source was not

mentioned in the Office Action. It must be clarified that the primer sequence TCCTTAATTTAAGGAATGTGA, specified in the paragraph 38 of the U.S. Publication No. 20030049627, is not sequence (g) as mentioned in Office Action p. 10; it is sequence (h). This sequence (h) (not g) is SEQ ID NO: 8 in U.S. Patent No. 6,924,102 and was selected based on a portion of exon 7 (between base pairs 901 and 921) of the SMN gene for the purpose of synthesizing probe 1 directed to exon 7 for the quantitative molecular diagnosis of SMA (Refer to page 8 of this Response -- section "Construction of Probes" from the summary of Nguyen's approach, U.S. Patent No. 6,924,102). If based on the CDS of the SMN gene, this same primer sequence (h) would be counted from base pairs 868 to base pairs 888. Regarding this present application, the counting of the selected instant primer sequence SEQ ID NO: 3 based on a portion of exon 7 is from base pairs 864 to 885 on the CDS (from exon 1 to a portion of exon 7) of the SMN gene, because the objective of this application's work is to get the SMN protein (295 amino acids) based on the CDS (885 bp) of the SMN gene. This explains the overlap concerning the sequences between the two primers, (h) and SEQ ID NO: 3.

The selection of primer sequence SEQ ID NO: 3 in this present application is not based on the primer sequence (h) of U.S. Patent No. 6,924,102. The reason for the selection of the primer sequence (h) and SEQ ID NO: 3 is based on the objective of each research work. In U.S. Patent No. 6,924,102, the objective is for quantitative molecular diagnosis of SMA based on the exons 7 and 8 of the SMN gene; in the present application, the objective is to get the SMN protein based on the CDS of the SMN gene.

As mentioned above, the objective of this application's work is to get the SMN protein based on the CDS (885 bp) of the SMN gene; this explains why the selected specific primers for RT (SEQ ID NO: 1) and PCR reactions (SEQ ID NOS: 2 and 3) are different from those of the

previous work specified in U.S. Patent No. 6,924,102 and U.S. Publication No. 20030049627. However the RT-PCR reaction conditions are similar:

-The RT reaction conditions used are the same (42°C for 45 minutes) in the previous work and the present application because the selected specific RT primers in both work have the same Tm (36°C).

-The PCR reaction conditions used are also the same because the selected specific PCR primers in both work have almost the same Tm (56°C and 58°C).

The number of cycles of 25 for PCR amplification in U.S. Patent No. 6,924,102 (and U.S. Publication No. 20030049627) was chosen because it was for SMA quantitative diagnostic purpose. In the present application, the high quantity of PCR products was the objective; therefore, the number of cycles of 35 for PCR amplification was selected.

*State of the art*

The state of the art can be summarized in terms of techniques in molecular biology such as the use of oligodT in RT reaction, the selection of specific primers for PCR amplification as well as the determination of the PCR reaction conditions as described by Wirth, Powell, Buck and Innis.

**Contribution over the art** related to claim 1

This applicant's contribution over the art is in:

-Selecting the specific primers for RT reaction (SEQ ID NO: 1) in order to get the specific SMN-cDNA containing the CDS of the SMN gene;

-Selecting the specific primers (SEQ ID NOS: 2 and 3), and optimizing the PCR reaction; conditions by taking into account the specific properties (base compositon, length and

concentration) of the target gene and primer sequences in order to get the PCR products corresponding to the CDS of the SMN gene;

-Determining the number of 35 cycles for PCR amplification in order to get high quantity of PCR products.

CLAIMS 2 and 3: OBTAINING THE EXPRESSION CONTRUCT OF THE HUMAN SMN GENE

Concerning claim 2, Office Action (p. 14-15) states that:

> "Melki teaches that the SMN gene may be expressed in "plasmids, cosmids, phages, YAC, pYAC, and the like". Melki further teaches expression in baculovirus cells.
>
> Melki teaches general methods for transferring the SMN gene between vectors comprising restriction digestion and ligation into a bacteriophage vector, and sequencing with M13 primers.
>
> However, Melki does not transfer the SMN gene from the pCR®II vector to the pFastBac vector followed by transformation into INVαF' and DH10Bac cells, colony screening and sequencing."

*Not a task for a person of ordinary skill in the art to perform*

The following explanation will demonstrate that this application's claim 2 is over Melki.

A. The very third sentence in the above mentioned statement in fact demonstrates that Melki does not teach what this **applicant has done in terms of conceiving and developing the method how to perform the ligation of the PCR product, cDNA of the CDS of the SMN gene (SMN-cDNA) into the pCR®II plamid vector in order to obtain vector (1) (pCR®II /SMN-cDNA).** In light of this procedure, a point of clarification must be noted here: At the time of this invention, the ligation reaction using ligase enzyme was actually needed for the construction of the vector (1) from the pCR®II TOPO vector of the Invitrogen TOPO TA cloning manual kit – which is

different from the new version of 2001 mentioned in the Office Action (p. 17) concerning the use of the pCR®II TOPO not requiring ligase.

B. What Melki does not teach – **the step for the construction of the vector (1) (pCR®II /SMN-cDNA) – is what this applicant has developed**; and this is crucial for the construction of the expression plasmids for SMN protein using the pFastBac™ HTb vector (Bac-to-Bac® Baculovirus Expression System), the pBlueBac His 2A, B, C vectors (Bac-N-Bac™ Baculovirus Expression System), and the pET-28a (+) vector (Prokaryotic Expression System). Indeed all of these expression vectors, pFastBac™ HTb, pBlueBac His 2A, B, C and pET-28a (+), have BamHI and XhoI restriction sites in their multiple cloning sites. The BamHI and XhoI restriction sites are present in the pCR®II vector. However, these restriction sites are absent in the cDNA of the CDS of the SMN gene (SMN-cDNA). Therefore, the issue is: How does one create the BamHI and XhoI restriction sites on the cDNA of the CDS of the SMN gene in order to ligate to the expression vectors pFastBac™ HTb, pBlue Bac His 2A, B, C and pET-28a (+)?

C. It is this applicant who resolved the above mentioned issue. This applicant performed the transfer of the SMN-cDNA to the pCR®II vector in which the BamHI and XhoI restriction sites are already present. Then, the applicant obtained the SMN-cDNA with the BamHI and XhoI restriction sites by digestion of vector (1) with BamHI and XhoI restriction enzymes. Then, this applicant took the SMN-cDNA with the obtained BamHI and XhoI restriction sites to ligate to the expression vector pFastBac™ HTb (Bac-to-Bac® Expression System), which thus results in vector (2) (pFastBac™ HTb/SMN-cDNA) to use for the expression of recombinant SMN

protein in insect cells (Refer to Figure 1). **Over the art is this applicant's creation of the Bam HI and Xho I restriction sites on the cDNA of the CDS of the SMN gene (SMN-cDNA) by selecting the commercially available pCR®II vector that has BamHI and XhoI. What is new is the fact that the applicant has identified this method which is the easiest and fastest way to get such a PCR product.** The state of the art has been the creation of the Bam HI and Xho I restriction sites on the cDNA of the CDS of the SMN gene obtained by performing the PCR amplification of the CDS of the SMN gene using the specific primers supplemented with the nucleotides corresponding to those of the Bam HI and Xho I restriction sites; this procedure is laborious because it requires the selection of such primers and the optimization of the PCR amplification in order to get such PCR product.

Next, to perform the construction of vector (**4**) (pBlue Bac His 2A) of the Bac-N-Bac™ Baculovirus Expression System to use for the expression of recombinant SMN protein in insect cells, the **applicant isolated the SMN-cDNA with the BamHI and XhoI restriction sites by digestion of vector (2) with BamHI and XhoI restriction enzymes in order to ligate to the expression vector pBlue Bac His 2A** (Refer to Figure 1).

D. Melki does not teach the use of INVαF' and DH10Bac cells, colony screening and sequencing. **Over Melki, the applicant deemed that INVαF' cells are ideal for propagating plasmids and cDNA library construction and used them for checking the success of the construction of the vectors (1), (2), (4) and (5).** The applicant used the DH10 Bac cells containing a parent bacmid DNA with a lac Z-mini-attTn fusion as a source of bamid needed for transfection of insect cells in the

next steps. The success of the transposition between the insert (SMN-cDNA) from the vector (**2**) (pFastBac™ HTb/SMN-cDNA) and the bacmid is visualized by the presence of the white bacterial colonies due to the expression cassette that disrupts the lac Z gene. The transfection of the insect cells is performed with the recombinant bacmid DNA.

E. Melki teaches expression constructs of the human SMN gene using a variety of different vectors, including baculovirus vectors, but does not specify a preferred baculovirus expression vector system (Office Action p. 17). **Over Melki, this applicant selected specifically the pFastBac and pBlue Bac His 2A vectors for obtaining a rapid and stable expression of recombinant SMN protein** (Office Action p. 18).

Concerning claim 3, Office Action (p. 19) states that Melki teaches many different expression systems including baculovirus and bacterial expression vectors; however Melki does not specify the pET-28a (+) bacterial expression vector. Melki does not teach the transfer of the SMN gene from the pFastBac vector followed by transformation into INVαF' cells and colony screening either.

*Not a task for a person of ordinary skill in the art to perform*

**Over Melki, this applicant selected the pET-28a (+) vector in order to get a rapid and stable production of a large amount of SMN recombinant protein.**

**Over Melki,** in order to perform the construction of vector (5) (pET-28a (+)/SMN-cDNA) (Prokaryotic Expression System) to use for the expression of recombinant SMN protein in E. Coli cells, this **applicant isolated the SMN-cDNA with the BamHI and XhoI restriction**

**sites by digestion of vector (2) with BamHI and XhoI restriction enzymes in order to ligate to the expression vector pET-28a (+).**

*State of the art*

The state of the art is the use of different expression vectors such as plasmids, cosmids, phages, YAC, pYAC and the like as described by Melki in order to perform the expression of SMN gene.

**Contribution over the art** related to claims 2 and 3

The applicant's contribution over the art is in:

-Developing the easiest and fastest way to get the SMN constructs by ligating the obtained SMN-cDNA products to the selected most powerful expression vectors commercially available;

-Developing the easiest and fastest way for creating the Bam HI and Xho I restriction sites on the obtained SMN-cDNA products by ligating them to the PCR® II vector commercially available, which result in vector (**1**);

-Developing the easiest and fastest way to get the SMN construct (**2**) from the obtained vector (**1**) by digestion of this vector (**1**) with Bam HI and Xho I restriction enzymes and by ligation of the resulting fragment to the expression plasmid vector pFastBac™ HTb which resulted in vector (**2**);

-Developing the easiest and fastest way to get the SMN constructs (**4**) and (**5**) from the obtained vector (**2**) by digestion of this vector (**2**) with Bam HI and Xho I restriction enzymes and by ligation of the resulting fragment to the expression plasmid vectors pBlue Bac His 2A and pET-28 a (+) which resulted in vectors (**4**) and (**5**) respectively;

-Utilizing the INVα F' cells for checking the success of the construction of the vectors (**1**), (**2**), (**4**) and (**5**).

**Conclusion**

This application is over the art because the inventor conceived and developed a new approach using 1) RT-PCR reactions in order to get the PCR products corresponding to the CDS of the SMN gene that encodes full-length SMN protein, and 2) commercially available expression vectors to get the different SMN constructs most easily and rapidly in order to rapidly produce a high yield of full-length recombinant SMN protein, capable of being purified via a simple, one-step affinity-based purification process. No one before has developed the easiest and fastest method of obtaining the SMN constructs via RT-PCR reactions and via the use of expression vectors commercially available.

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